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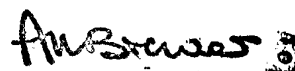
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4. Title of the invention

"Stem Cells"

5. Name of your agent (if you have one)

Murgitroyd & Company

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Scotland House
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12. Name and daytime telephone number of person to contact in the United Kingdom

Douglas Drysdale

0141 307 8400

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1 Stem Cells

2

3 The present invention relates to the culture of
4 primate embryonic stem cells, to the provision of
5 feeder cells of human origin to support embryonic
6 stem cell culture, and to the provision of
7 fibroblast cells for therapeutic use.

8

9 Embryonic stem cells are undifferentiated cells
10 able to proliferate for long periods and which can
11 be induced to differentiate into any type of adult
12 cell.

13

14 Human embryonic stem (hES) cells represent a great
15 potential source of various cell types for
16 therapeutic uses, pharmokinetic screening and
17 functional genomics applications (Odorico et al.,
18 2001, Stem Cells 19:193-204; Schuldiner et al.,
19 2001, Brain Res 913:201-205; Zhang et al., 2002,
20 Nat Biotechnol 19:1129-1133; He et al., 2003, Circ
21 Res 93:32-39).

22

1 Typically embryonic stem cells are obtained from an
2 embryo at the blastocyst stage (5 to 7 days), by
3 extraction of the inner cell mass (ICM). The ICM
4 is a group of approximately 30 cells located at one
5 end of the internal cavity of the blastocyst.
6 Pluripotent hES cell lines have been obtained from
7 the ICM of Day 5 to 7 blastocysts (Thomson et al.,
8 1998, Science 282:1145-1147; Reubinoff
9 et al., 2000 Nature Biotechnol 18:399-404; Richards
10 et al., 2002, Nature Biotechnol 20:933-936; Hovatta
11 et al., 2003, Hum Reprod 18:1404-1409; Mitalipova
12 et al., 2003, Stem Cells 21:521-526) but to date
13 there have been no reports of obtaining hES cells
14 from older blastocysts due to the difficulty of
15 maintaining the viability of the blastocysts *in*
16 *vitro*.

17
18 Continuous culture of embryonic stem cells in an
19 undifferentiated (pluripotent) state requires the
20 presence of feeder layers such as mouse embryonic
21 fibroblast (MEF) cells (Thomson et al., 1998,
22 Science 282:1145-1147; Reubinoff et al., 2000, Nat
23 Biotechnol 18:399-404), STO cells (Park et al.,
24 2003, Bio Reprod 69:2007-2017), human foreskin
25 fibroblasts (Hovatta et al., 2003, Hum Reprod
26 18:1404-14069) human adult fallopian tubal
27 epithelial cells, human fetal muscle and human
28 fetal skin cells (Richards et al. 2002, Nature
29 Biotechnol 20:933-935), or adult skin fibroblast
30 cell lines (Richards et al. 2003, Stem Cells
31 21:546-556). Alternatively, the culture media can
32 be conditioned by growing the feeder cells in the

1 medium and then harvesting the medium for
2 subsequent stem cell culture (see WO-A-99/20741).
3 Whilst this method is referred to as "feeder-free"
4 culture, nonetheless there is still a reliance on
5 the feeder cells to culture isolated ICMs and to
6 condition the media and hence there is potential
7 for pathogen transmission.

8

9 Unfortunately the use of feeder cells for the
10 culture of hES cells limits their medical
11 application for several reasons: xenogeneic and
12 allogeneic feeder cells bear the risk of
13 transmitting pathogens and other unidentified risk
14 factors (Richards et al., 2002, Nat Biotechnol
15 20:933-936; Hovatta et al., 2003, Hum Reprod
16 18:1404-1409). Also, not all human feeder cells
17 and cell-free matrices support the culture of hES
18 cells equally well (Richards et al., 2002, Nat
19 Biotechnol 20:933-936; Richards et al., 2003, Stem
20 Cells 21: 546-556), and the availability of human
21 cells from aborted fetuses or Fallopian tubes is
22 relatively low. Additionally there are ethical
23 concerns regarding the derivation of feeder cells
24 from aborted human fetuses.

25

26 For example, WO-A-03/78611 describes a method of
27 culturing human fibroblasts delivered from aborted
28 human fetuses, typically of 4 to 6 week gestation.
29 The fibroblasts are cultured from the rib region of
30 the embryo and are described as being suitable to
31 support human embryonic stem cell culture. However

1 this method relies upon the donation of aborted
2 fetuses to maintain a supply of fibroblasts.
3 US-A-2002/0072117 and US 6,642,048 describe the
4 production of a human embryonic stem cell line by
5 culturing the ICM of blastocysts and subsequently
6 inducing the embryonic stem cells to form embryoid
7 bodies and to differentiate into a mixed
8 differentiated cell populations. Cells having a
9 morphology typical of fibroblasts were selected for
10 use as feeder layers or to condition cell culture
11 media for feeder-free culture. However no markers
12 typical of fibroblasts were noted as being present
13 on these cells.

14
15 There remains a need to culture primate embryonic
16 stem (pES) cells, especially hES cells intended for
17 therapeutic use, using only feeder cells of the
18 same species or media conditioned by such feeder
19 cells, to reduce the risk of cross-species pathogen
20 transmission. Additionally, as mentioned above,
21 the use of aborted fetuses as a source of human
22 feeder cells is recognised to be of ethical concern
23 and an alternative source of suitable feeder cells
24 is required.

25
26 The present invention provides a novel human
27 embryonic stem (hES) cell line. The novel cell
28 line is termed hES-NCL1. A sample of the hES-NCL1
29 cell line was deposited in accordance with the
30 Budapest Treaty on 13 January 2005 at the National
31 Institute for Biological Standards and Control
32 (NIBSC), Blanche Lane, South Mimms, Potters Bar

1 Herts., EN6 3QC. The accession Number allocated to
2 the sample was P-05-001.

3

4 The hES cell line described above was isolated
5 using novel methodology, which forms a further
6 aspect of this invention, and was noted to
7 spontaneously differentiate into fibroblast-like
8 cells in the absence of any trigger and without the
9 formation of embryoid bodies. The fibroblast-like
10 cells so formed expressed the specific fibroblast
11 marker AFSP (anti-fibroblast cell surface specific
12 protein, from Sigma). A photomicrograph of the
13 stained fibroblast-like cells is shown at Figures
14 2B, C, D. The stem cell derived fibroblast-like
15 cells, their formation and their use in culture (as
16 feeder cells or to condition the culture media) of
17 animal embryos (including non-human embryos such as
18 non-human primate embryos as well as human embryos)
19 or embryonic or non-embryonic stem cells (which
20 embryonic or non-embryonic stem cells may be of
21 human or non-human origin), and in therapy forms a
22 further aspect of the present invention and is
23 discussed further below.

24

25 In one aspect, the present invention provides a
26 method of culturing a blastocyst, said method
27 comprising exposing said blastocyst to Buffalo rat
28 liver cells or media conditioned thereby for at
29 least 12 hours.

30

1 The Buffalo rat liver cells may conveniently be
2 present in the cell culture media or, more
3 preferably, will be used to condition that media.

4

5 The blastocyst may be exposed to the Buffalo rat
6 liver cells or media conditioned thereby for a
7 minimum period of 24 hours, 36 hours, 48 hours, 60
8 hours or 72 hours. We have found that an exposure
9 period of approximately 2 days is sufficient.

10 Where the blastocyst is to be used to generate
11 pluripotent embryonic stem cells, it is desirably
12 exposed to the Buffalo rat liver cells or media
13 conditioned thereby in the period immediately prior
14 to the extraction of cells of the ICM. Benefits
15 may also be obtained from exposing the blastocyst
16 to Buffalo rat liver cells or media conditioned
17 thereby where it is intended for preimplantation as
18 part of IVF treatment.

19

20 In more detail, one protocol for culturing a
21 blastocyst according to the present invention
22 comprises:

- 23 i) culturing said blastocyst from fertilisation
24 in G1 media;
- 25 ii) transferring said blastocyst of step i) to
26 G2.3 media and maintaining said blastocyst in
27 the G2.3 media; and
- 28 iii) transferring said blastocyst of step ii) to
29 cell culture media conditioned by Buffalo rat
30 liver cells.

31

1 The G1 and G2.3 media referred to above can be
 2 obtained from Vitrolife Sweden AB, Kungsbacka,
 3 Sweden.

4
 5 G-1™ is a media designed to support the
 6 development of embryos to the 8-cell stage, ie.
 7 from pro-cleavage to day 2 or 3. The media
 8 contains carbohydrates, amino acids and chelators,
 9 as well as Hyaluronan and is bicarbonate buffered.
 10 In more detail, the G-1™ media contains:

11 Alanine	Penicillin G
12 Alanyl-glutamine	Potassium chloride
13 Asparagine	Proline
14 Aspartate	Serine
15 Calcium chloride	Sodium bicarbonate
16 EDTA	Sodium chloride
17 Glucose	Sodium dihydrogen phosphate
18 Glutamate	Sodium lactate
19 Glycine	Sodium pyruvate
20 Hyaluronan	Taurine
21 Magnesium sulphate	Water for injection (WFI)

22
 23 G-2™ is a cell culture media to support the
 24 development of embryos from around the 8-cell stage
 25 to the blastocyst stage. The media contains
 26 carbohydrates, amino acids and vitamins, as well as
 27 Hyaluronan, and is bicarbonate buffered. In more
 28 detail the G-2™ version 3 (ie. G2.3) media
 29 contains:

31 Alanine	Penicillin G
32 Alanyl-glutamine	Phenylalanine

1	Arginine	Potassium chloride
2	Asparagine	Proline
3	Aspartate	Pyridoxine
4	Calcium chloride	Riboflavin
5	Calcium pantothenate	Serine
6	Cystine	Sodium bicarbonate
7	Glucose	Sodium chloride
8	Glutamate	Sodium dihydrogen phosphate
9	Glycine	Sodium lactate
10	Histidine	Sodium pyruvate
11	Hyaluronan	Thiamine
12	Isoleucine	Threonine
13	Leucine	Tryptophan
14	Lysine	Tyrosine
15	Magnesium sulphate	Valine
16	Methionine	Water for injection (WFI)

17

18 The duration of step i) above may typically be from
 19 Day 0 (at fertilisation) to Day 3.

20

21 The duration of step ii) above may typically be for
 22 2 or 3 days, that is from Day 3 to Day 5 or 6.

23

24 The duration of step iii) above is for a minimum
 25 period of 24 hours as described above, but may
 26 typically be for 1 to 3 days.

27

28 In step iii) a preferred cell culture media
 29 consists of Dulbecco's modified Eagle's medium
 30 (DMEM, Invitrogen, Paisley, Scotland), optionally
 31 supplemented with 15% (v/v) Glasgow medium, and
 32 conditioned by Buffalo rat liver cells (see

1 Stojkovic et al., 1995, Biol Reprod 53:1500-1507).
2 Typically conditioning by the Buffalo rat liver
3 cells comprises culturing 75000 buffalo rat liver
4 cells/cm² in Glasgow medium for 24-36 hours. The
5 media is then recovered and frozen at -20°C until
6 required.

7

8 Using a blastocyst cultured as described above, the
9 ICM can be extracted using routine techniques as
10 late as Day 8, typically by immunosurgery (see
11 Reubinoff et al., 2001, Hum Reprod 10:2187-2194).
12 Blastocysts were cultured for 30 minutes in whole
13 human antiserum (Sigma) diluted 1:5 in DMEM+FCS
14 medium (i.e. 80% Dulbecco's modified Eagle's medium
15 with 10-20% (v/v) fetal calf serum). Furthermore,
16 the blastocysts were washed three times and
17 cultured for another period of approximately 20
18 minutes in guinea pig complement (1:5). The
19 isolated ICMs were used for embryonic stem cell
20 culture but could alternatively be implanted into a
21 receptive female as part of an IVF treatment.

22

23 For human blastocysts, the blastocyst will have
24 been donated, with informed consent, as being
25 superfluous to IVF treatment. For other (ie. non-
26 human) primates, the ovulation cycle can be
27 controlled by intramuscular injection of
28 prostaglandin or a prostaglandin analogue, and the
29 embryos harvested by a non-surgical uterine flush
30 procedure (see Thompson et al., 1994, J Med
31 Primatol 23:333-336) at day 8 following ovulation.

32

1 If the blastocyst is unhatched, the zona pellucida
2 is removed by brief exposure to pronase. This step
3 is not required for hatched embryos. The
4 blastocyst is exposed to antiserum for 30 minutes.
5 The blastocyst is then washed three times in DMEM,
6 and exposed to a 1:5 dilution of Guinea pig
7 complement (Gibco) for 20 minutes. After two
8 further washes in DMEM, lysed trophectoderm cells
9 are removed from the ICM by pipette and the ICM
10 plated out on a suitable feeder layer. Embryonic
11 stem cell lines are identified from the cultured
12 ICM cells.

13

14 As mentioned above, the novel methodology enables
15 the blastocyst to be cultured at a relatively late
16 stage, day 8. At day 8 the number of cells
17 obtainable from the ICM is considerably increased,
18 but surprisingly these cells retain their
19 pluripotent ability.

20

21 The present invention therefore provides a method
22 of producing an embryonic stem cell line, said
23 method comprising:

- 24 i) culturing a blastocyst as described above; and
25 ii) extracting cells of the ICM from said
26 blastocyst and culturing the cells to produce
27 an embryonic stem cell line therefrom.

28

29 The reference to culturing the cells of the ICM
30 extracted from the blastocyst in step ii) above
31 includes the published protocols available and is

1 not especially dependent upon any particular
2 culture conditions.

3
4 The method of producing stem cells according to the
5 present invention provides a generic and efficient
6 method for the production of primate embryonic stem
7 (pES) cell lines. The pES cell lines may be human
8 embryonic stem (hES) cell lines. Alternatively the
9 pES cells may be of non-human origin. The stem
10 cell lines so produced are preferably of clinical
11 and/or GMP grade.

12
13 One suitable medium for the isolation of embryonic
14 stem cells consists of 80% Dulbecco's modified
15 Eagle's medium (DMEM; obtainable from Invitrogen or
16 Gibco) with 10-20% (v/v) fetal calf serum (FCS,
17 Hyclone, Logan, UT). Optionally the medium may
18 also include one or more of 0.1 mM β -
19 mercaptoethanol (Sigma), up to 1% (v/v) non-
20 essential amino acid stock (Gibco), 1% (v/v)
21 antibiotic, such as penicillin-streptomycin
22 (Invitrogen), and/or 4ng/ml bFGF (Invitrogen). To
23 date details of several specific media suitable for
24 embryonic stem cell culture have been published in
25 the literature - see for example Thomson et al.,
26 1998, Science 282:1145-1147; Xu et al., 2001,
27 Nature Biotechnol 19:971-974; Richards et al.,
28 2002, Nature Biotechnol 20:933-936; and Richards et
29 al., 2003, Stem Cells 21:546-556.

30
31 Feeder cells which may be used for stem cell
32 culture include mouse embryonic stem cells (MEF),

1 STO cells, foetal muscle, skin and foreskin cells,
2 adult Fallopian tube epithelial cells (Richards et
3 al., 2002, Nat Biotechnol 20:933-936; Amit et al.,
4 2003, Biol Reprod 68:2150-2156; Hovatta et al.,
5 2003, Hum Reprod 18:1404-1409; Park et al., 2003,
6 Biol Reprod 69, 2007-2014; Richards et al., 2003,
7 Stem Cells 21:546-556), adult bone marrow cells
8 (Cheng et al., 2003, Stem Cells 21:131-142), or on
9 coated dishes with animal based ingredients with
10 the addition of MEF cell conditioned media (Xu et
11 al., 2001, Nature Biotechnol 19:971-974).

12
13 The method of culturing a blastocyst and the method
14 of producing embryonic stem cell lines as described
15 above are both suitable for use with blastocysts of
16 primate origin, including blastocysts of human or
17 non-human origin.

18
19 The human embryonic stem cells of the present
20 invention are characterised by at least one of the
21 following;

- 22 i) presence of the cell surface markers TRA-1-60,
23 GTCM2, and SSEA-4;
24 ii) expression of *Oct-4*;
25 iii) expression of *NANOG*;
26 iv) expression of *REX-1*; and/or
27 v) expression of *TERT*.

28
29 In one embodiment at least 2 or more of the
30 characteristics listed above are present,
31 preferably 3 or more of the characteristics are
32 present, especially 4 or more, more preferably all

1 of the above characteristics are present in the
2 stem cells.

3

4 The antigen SSEA-4 is a glycolipid cell marker.
5 Specific antibodies to identify this marker are
6 available from the Development Studies Hybridoma
7 Bank, DSHB, Iowa City, IA.

8

9 The cell surface marker TRA-1-60 is recognised by
10 antibodies produced by hybridomas developed by
11 Peter Andrews of the University of Sheffield (see
12 Andrews et al., "Cell lines from human germ cell
13 tumours" pages 207-246 in Teratocarcinomas and
14 Embryonic Stem Cells: A Practical Approach, Ed.
15 Robertson, Oxford, 1987). TRA1-60 is also
16 commercially available (Chemicon). Both GTCM2 and
17 TG343 are described in Cooper et al., 2002, J.
18 Anat. 200(Pt 3):259-65.

19

20 The embryonic stem cell line produced according to
21 the method of the present invention as described
22 above (and specifically the stem cell line hES-
23 NCL1) can be used for screening and/or to produce
24 differentiated cells of specific cell types for
25 therapeutic purposes (e.g. for implantation to
26 replace damage or missing tissue). The stem cell
27 lines (e.g. hES-NCL1) can be used to screen agents
28 (e.g. chemical compounds or compositions) for
29 toxicity and/or for therapeutic efficacy (i.e.
30 pharmacological activity).

31

1 In a further aspect, the present invention provides
2 a method of screening an agent for toxicity and/or
3 for therapeutic efficacy, said method comprising:

- 4 a) exposing an embryonic stem cell line
5 obtained according to the method described
6 (e.g. hES-NCL1) to said agent;
7 b) monitoring any alteration in viability
8 and/or metabolism of said stem cells; and
9 c) determining any toxic or therapeutic effect
10 of said agent.

11
12 Additionally, the method of producing a stem cell
13 line according to the present invention as
14 described above, and the stem cell lines produced
15 thereby (e.g. hES-NCL1) may be used in the creation
16 of an embryonic stem cell bank for use in screening
17 and/or to produce differentiated cells of specific
18 cell types for therapeutic purposes. The stem cell
19 bank, which forms a further aspect of the present
20 invention, will consist of a multiplicity of
21 genetically distinct stem cell lines. The stem
22 cell lines forming the stem cell bank will usually
23 be of primate embryonic stem cells such as human
24 embryonic stem cells or non-human embryonic stem
25 cells. The embryonic stem cell bank can be used to
26 screen agents (e.g. chemical compounds or
27 compositions) for toxicity and/or for therapeutic
28 efficacy (i.e. pharmacological activity).

29
30 Thus, in a yet further aspect, the present
31 invention provides a method of screening an agent

1 for toxicity and/or for therapeutic efficacy, said
2 method comprising:

- 3 a) exposing an embryonic stem cell bank
4 comprising a multiplicity of embryonic stem
5 cell lines obtained according to the method of
6 the present invention to said agent;
7 b) monitoring any alteration in viability and/or
8 metabolism of said stem cells; and
9 c) determining any toxic or therapeutic effect of
10 said agent.

11
12 As briefly mentioned above, it was noted that the
13 embryonic stem cell line established from a
14 blastocyst cultured as described above according to
15 the present invention spontaneously differentiated
16 into fibroblast-like cells without formation of
17 embryoid bodies. Such spontaneous differentiation
18 into a single cell type was unexpected. These
19 fibroblast-like cells then acted as a feeder layer
20 for the remaining undifferentiated embryonic stem
21 cells of the culture. The stem cell derived
22 fibroblast-like cells and the embryonic stem cells
23 supported thereby were autogeneic.

24
25 The spontaneous differentiation of hES cells in a
26 feeder-free culture into a mixture of cell types,
27 including fibroblast-like cells, has already been
28 described (see Park et al., 2003, Biol Reprod
29 69:2007-2014) but in that study the differentiation
30 was observed in the centre of the hES cell
31 colonies. This differs to the present invention
32 where differentiation occurs at the periphery of

1 the colony. Moreover in the present invention only
2 fibroblast-like cells were observed and no other
3 cell types were noted to be present.

4

5 The present invention therefore provides a method
6 of producing fibroblast-like cells, said method
7 comprising:

- 8 i) culturing a blastocyst as described above;
9 ii) extracting cells of the ICM from said
10 blastocyst and culturing the cells to produce
11 an embryonic stem cell line therefrom; and
12 iii) allowing cells of said embryonic stem cell
13 line to differentiate into stem cell derived
14 fibroblast-like cells.

15

16 The stem cell derived fibroblast-like cells are
17 produced without requiring a specific stimulant,
18 e.g. growth factor or change in physical growth
19 conditions (e.g. allowing the cells to become
20 crowded).

21

22 One suitable method for obtaining differentiation
23 of the stem cells into fibroblast-like cells was
24 simply to transfer the stem cells to cell culture
25 media in the absence of feeder cells or feeder cell
26 conditioning. The stem cells responded by
27 differentiation of a proportion of the stem cells
28 which then acted as feeder cells for the non-
29 differentiated remaining stem cells. Thus
30 obtaining differentiation into fibroblast-like
31 cells was possible using an extremely easy one-step
32 process, avoiding the need for time-consuming

1 procedures and allowing the differentiation to be
2 fully controlled under *in vitro* conditions.

3
4 The stem cell derived fibroblast-like cells are
5 characterised by a morphology typical of the cell
6 type, ie. long flat cells with an elongated,
7 condensed nucleus. The cytoplasmic processes
8 therein resemble those found in fibroblasts of
9 connective tissue.

10
11 The fibroblast-like cells of the present invention
12 are positive for the cell surface marker AFSP. In
13 addition, the identity of hES cells-derived
14 fibroblasts was confirmed by karyotyping and DNA
15 analysis of both stem cells and hES cells-derived
16 fibroblasts. This confirmed that hES cells-derived
17 fibroblasts are autogeneic i.e. of the same origin
18 as the stem cells.

19
20 The fibroblast-like cells according to the present
21 invention could be easily immortalised using known
22 techniques to provide a long term source of the
23 cells.

24
25 The present invention also provides a novel human
26 embryonic stem cell derived fibroblast-like cell
27 line. The novel fibroblast-like cell line, termed
28 hESCdF-NCL, has been deposited at the European
29 Collection of Cell Cultures on 19 January 2004
30 under Accession No 04010601.

31

1 The fibroblast-like cells and media conditioned by
2 the fibroblast-like cells of the present invention
3 are suitable to support the growth of embryos. The
4 fibroblast-like cells and media conditioned by the
5 fibroblast-like cells of the present invention are
6 alternatively suitable to support the growth of
7 stem cells, especially non-human primate embryonic
8 stem cells or human embryonic stem cells. Other
9 types of stem cells needing the use of feeder cells
10 to survive are also included and particular mention
11 may be made of unipotential and pluripotential stem
12 cells such as adult stem cells, haemopoietic stem
13 cells, mesenchymal stem cells, osteogenic stem
14 cells, chondrogenic stem cells, neuronal stem
15 cells, gonadal stem cells, epidermal stem cells and
16 somatic/progenitor stem cells. Where the
17 fibroblast-like cells of the present invention are
18 used to support human stem cells, the fibroblast-
19 like cells are desirably autogeneic thereto but
20 xenogeneic feeder cells may be used following
21 screening to ensure that they are pathogen-free.

22
23 In a further aspect, the present invention provides
24 a self-feeder system for the growth of
25 undifferentiated stem cells, said system comprising
26 i) culturing a blastocyst as described above;
27
28 ii) extracting cells of the ICM from said
29 blastocyst and culturing the cells to produce
30 an embryonic stem cell line therefrom; and
31

1 iii) and allowing some of the cells of said
2 embryonic stem cell line to differentiate
3 into stem cell derived fibroblast-like cells
4 whilst the remainder of the cells of said
5 embryonic stem cell line remain in an
6 undifferentiated, pluripotent state, whereby
7 said stem cell derived fibroblast-like cells
8 act as autogeneic feeder cells for said stem
9 cells.

10

11 The fibroblast-like cells may be used directly as
12 feeder cells to support stem cell culture (eg are
13 grown as a confluent surface in contact with the
14 stem cells) or may be used to condition media for
15 use in stem cell culture. Generally, where the
16 media is to be conditioned, the fibroblast-like
17 cells are grown in the media for a predetermined
18 period of typically 24 hours, although periods of
19 up to a maximum of 9 days may be used, before the
20 media is removed and transferred to the stem cells.

21

22 There are several advantages for using hES cells
23 derived fibroblasts as feeder cells: i) feeder
24 derived from hES cells offers more secure
25 autogeneic/genotypically homogenous system for
26 prolonged growth of undifferentiated hES cells, ii)
27 feeders differentiated from first clinical-grade
28 hES cell line could be used worldwide as initial
29 monolayer for growth of isolated ICMS to eliminate
30 transfer of pathogens, iii) the long proliferation
31 time of already derived hES cell lines allows
32 screening for viral contamination, iv) medium

1 conditioned by hESdF can be used for feeder-free
2 growth of hES cells thus avoiding potential viral
3 transfer from the MEF conditioned media used to
4 date, v) due to the low bioburden, embryonic
5 tissues perform better support *in vitro* than adult
6 tissues (see Richards et al., 2003, Stem Cells
7 21:546-556), vi) derivation and culture of hESdF is
8 fully controlled and not time consuming, vii)
9 derived feeder cells could be easily immortalized
10 to provide a long-term source of this tissue, viii)
11 *in vitro* studies on cell-to-cell contacts and
12 identification of isolated soluble factors could
13 significantly improve cell-culture, cell-
14 transplantation and tissueengineering avoiding at
15 the same time expensive tissue-biopsy and
16 unnecessary sacrifice of animals.

17
18 Accordingly, the present invention further provides
19 a method of culturing a primate embryonic stem cell
20 line, such as a human embryonic stem cell line, to
21 maintain the viability of eggs prior to or during
22 fertilisation and/or to culture blastocysts or
23 embryos intended for implantation into a receptive
24 female to establish a pregnancy (i.e. as part of an
25 IVF procedure). The method comprises providing
26 fibroblast-like cells obtained according to the
27 present invention as feeder cells or to condition
28 the cell culture media. Advantageously the
29 fibroblast-like cells selected will be obtained
30 from an embryonic stem cell line of the same origin
31 or species, and will be previously screened to
32 ensure pathogen-free status. This approach enables

1 the complete elimination of animal ingredients for
2 the culture of undifferentiated hES cells and
3 avoids the potential of viral transfer which may
4 occur when MEF conditioned media or conditioned
5 media from other feeders is used for stem cell
6 culture.

7
8 We have found that the use of the fibroblast-like
9 cells obtained according to the present invention
10 (e.g. hESCdF-NCL) as feeder cells or to condition
11 the culture media enables the undifferentiated
12 culture of the embryonic stem cells. It is
13 anticipated that a similar ability will be obtained
14 using other stem cell types. This is highly
15 significant for the long term maintenance of such
16 cell lines and also has the advantage that the
17 extended culture period possible for the
18 undifferentiated stem cell line enables the cell
19 line to be screened for any potential pathogen
20 (e.g. viral contamination).

21
22 Alternatively, the fibroblast-like cells can be
23 used for therapy, for example to assist
24 regeneration of wounds requiring fibroblast
25 presence.

26
27 The presence of fibroblast cells, without
28 contamination of other cell types is of particular
29 advantage in therapy. One example of the use of
30 the fibroblasts according to the present invention
31 is the generation of skin grafts for use in

treating wounds (for example burns) or in cosmetic or regenerative surgery.

The present invention will now be further described with reference to the following examples and figures, in which:

Figure 1. Morphology of human blastocysts and hES cells. Day 6 blastocysts (A) and hatched Day 8 blastocysts (B). Note the presence of very well organised inner cell mass in Day 8 blastocyst recovered after three-step *in vitro* culture. Inner cell mass cells (C) grown on irradiated MEF 4 days after immunosurgery. Primary hES cells colony (D) grown on inactivated MEF cells. Same colony at high magnification (E). Bars: 50 μm (A-D); 100 μm (E).

Figure 2. Morphology and characterisation of hES cells-derived fibroblasts. Undifferentiated hES cells (A). Peripheric differentiation of hES cells into fibroblast-like cells in feeder-free conditions (B). Phase (C) and fluorescence (D) microscopy of hES cells-derived fibroblasts using AFSP antibody. Normal 46 + XX karyotypes of hES cells (E) and hES cells-derived fibroblasts (F). Microsatellite analysis of hES cells (G) and hES cells-derived fibroblasts (H). Bars: 50 μm (A, C, D), 100 μm (B).

Figure 3. Morphology of frozen/thawed hES-NCL1 colony cultured on frozen/thawed hES cell-derived fibroblasts. Bar: 50 μm .

1
2 **Figure 4.** Morphology and characterisation of hES-
3 NCL1 cells grown on γ -irradiated hESdF monolayer
4 (A-F) or feeder-free (G, H). (A) Five days old
5 vitrified hES-NCL1 colony cultured on frozen/thawed
6 hESdF (passage 8). (B) Higher magnification of the
7 same hES colony. Note typical morphology of hES
8 cells i.e. small cells with prominent nucleoli. HES
9 cells grown on hESdF stained with antibody
10 recognising the TRA1-60 (D) and SSEA-4 (F)
11 epitopes. HES cells grown on Matrigel (G) with
12 addition of hESdF conditioned medium stained with
13 antibody recognising the GTCM2 epitope (H). Bars:
14 200 μ m (A, E-H); 50 μ m (B); 100 μ m (C, D).
15

16 **Figure 5.** Characterisation and karyotyping of hES-
17 NCL1 cells grown on hESdF monolayer. RT-PCR
18 analysis of undifferentiated hES cells grown on
19 inactivated hESdF cells (A). PCR products obtained
20 using primers specific for *OCT-4*, *NANOG*, *FOXD3*,
21 *TERT*, *REX1* and *GAPDH*. HES cells (passage 31) grown
22 on hESdF (passage 11) show normal female karyotype
23 (46, XX) (B).
24
25

26 **Figure 6.** Histological analysis of teratomas formed
27 from grafted colonies of hES cells grown on
28 inactivated hESdF in testis (A-C) and kidney (D-F)
29 of SCID mice. (A) neural epithelium (ne); (B)
30 aggregation of glandular cells with characteristic
31 appearance of secretory acini (sa); (C) cartilage
32 (cart); (D) wall of respiratory passage showing

1 epithelium (ep), submucosa (sm), submucosal glands
 2 (sg). Epithelium contains occasional ciliated cells
 3 and numerous goblet cells secreting mucin (m); (E)
 4 Two types of epithelia: respiratory (top),
 5 keratinised skin (bottom). Submucosal glands (sg)
 6 located beneath pseudostratified ciliated (in
 7 parts) epithelium (ep). Structures of the skin
 8 include epidermis (ed), dermis (dm) and cornified
 9 layer (c). Note that the stratum granulosum (arrow)
 10 is characterised by intracellular granules which
 11 contribute to the process of keratinisation.
 12 Occasional mitotic indices (m) are seen in the
 13 basal layer; (F) High magnification image of skin,
 14 showing greater detail of dermis (dm), epidermis
 15 (ed) and cornified layer (c). Again the stratum
 16 granulosum is visible (arrow). Scale bars: (A, B,
 17 C) 100 μm ; (D, E) 25 μm ; (F) 17.5 μm .

18
 19 **Figure 7.** Flow cytometry analysis of hESdF (left
 20 panel) and human foreskin fibroblasts (HFF, right
 21 panel) for the presence of CD31, CD44, CD71, CD90
 22 and CD106. The bold (red) line represents the
 23 staining with the isotype control and the grey
 24 (green) line staining with specific antibodies.

25
 26 **Figure 8.** Spontaneous differentiation of hES-NCL1
 27 cells grown on hESdF and then in feeder-free
 28 conditions. hES-NCL1 differentiate into neuronal
 29 (A) and smooth muscle (B) cells demonstrating
 30 differentiation into cells of ectoderm and
 31 mesoderm, respectively. Green: cells stained with
 32 nestin antibody (A) and smooth muscle actin
 33 antibody (B). Red: cell-nuclei stained with

propidium iodide. (A) shows small areas of red and green staining dispersed across the cells in a check-like pattern. (B) shows all cells stained green. Scale bars: 100 μm (A) and 50 μm (B).

Examples

Material and Methods

Culture of embryos. Two day old human embryos, produced by *in vitro* fertilization (IVF) for clinical purposes, were donated by individuals after informed consent and after Human Fertilisation and Embryology Authority (HFEA, UK) approval. Until Day 3 (IVF = Day 0), 11 embryos were cultured in G1 medium and transferred to G2.3 medium (both G1 & G2.3 from Vitrolife, Kungsbacka, Sweden) until day 6. Day 6 recovered blastocysts were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Paisley, Scotland) supplemented with 15% (v/v) Glasgow medium conditioned by Buffalo rat liver cells which has been used successfully for the long-term culture of bovine embryos, termed G-BRLC media (Stojkovic et al., 1995, Biol Reprod 53:1500-1507). On Day 8 ICMS were isolated by immunosurgery as previously described (Reubinooff et al., 2001, Hum Reprod 10:2187-2194).

Cell-number analysis. We investigated whether our three-step embryo culture supported development of

1 Day 8 blastocysts and whether these blastocysts
2 posses more ICM cells than Day 6 blastocysts.
3 Eleven isolated ICMs from Day 6 blastocysts (5
4 blastocysts and 6 expanded blastocysts) and 13 ICMs
5 from Day 8 blastocysts (7 expanded and 6 hatching
6 or hatched blastocysts) were analysed using 1.5
7 µg/ml 4'-6-diamidino-2-phenylindole (DAPI, Sigma,
8 St. Louis, MO) labelling as previously described
9 (Spanos et al., 2000, Biol Reprod 63:1413-1420).

10

11 **Derivation of hES cells.** Initially, isolated ICMs
12 were cultured on γ-irradiated MEFs monolayer
13 (75.000 cell/cm²) and DMEM supplemented with 10%
14 (v/v) Hyclone defined fetal calf serum (FCS,
15 Hyclone, Logan, UT) for 10 days. After 17 days, the
16 hES cell colony was mechanically dispersed into
17 several small clumps which were cultured on a fresh
18 MEF layer with ES medium containing Knockout-DMEM
19 (Invitrogen), 100 µM β-mercaptoethanol (Sigma), 1
20 mM L-glutamine (Invitrogen), 100 mM non-essential
21 amino acids, 10% serum replacement (SR,
22 Invitrogen), 1% penicillin-streptomycin
23 (Invitrogen) and 4 ng/ml bFGF (Invitrogen). ES
24 medium was changed daily. Human embryonic stem
25 cells were passaged by incubation in 1 mg/ml
26 collagenase IV (Invitrogen) for 5-8 minutes at 37°C
27 or mechanically dissociated and then removed to
28 freshly prepared MEF or hES cells-derived feeders.

29

30 **Recovery of hES cell-derived fibroblasts.** Once a
31 stable stem cell line was established, hES cells
32 were transferred into feeder-free T-25 flasks:

1 (Iwaki, Asahi, Japan), using DMEM supplemented with
2 10% FCS at 37°C in a 5% CO₂ atmosphere. After one
3 week the stem cell derived fibroblast-like cells
4 were transferred into T-75 flasks (Iwaki) and
5 cultured for a further 3 days to produce a
6 confluent primary monolayer of hES cells-derived
7 fibroblasts.

8
9 **Immunocytochemical analysis of hES cells and hES**
10 **cells-derived fibroblasts.** Live staining was
11 performed by adding primary antibodies (TRA1-60 and
12 TRA1-81, a kind gift from Prof. P. Andrews
13 (University of Sheffield, UK) (but also available
14 commercially from Chemicon); SSEA-4, SSEA-4 (MC-
15 813-70) from Developmental Studies Hybridoma Bank,
16 DSHB, Iowa City, IA; GCTM-2 and TG343, both a kind
17 gift from Dr. M. Pera (Monash Institute of
18 Reproduction and Development, Clayton, Australia);
19 anti-fibroblast surface protein, AFSP from Sigma)
20 to hES cells and hES cells-derived fibroblasts for
21 20 minutes at 37°C. The primary antibodies were
22 used at the following dilutions: TRA-1-60 - 1:10;
23 TRA1-81 - 1:10; SSEA-3 - 1:4; SSEA-4 - 1:5
24 (Henderson et al., 2002, Stem Cells 20:239-337);
25 GCTM-2 - 1:2; AFSP - 1:50 (Ronnov-Jessen, 1992,
26 Histochem Cytochem 40:475-486). TG343 at 1:2
27 (Cooper et al., 2002, J Anat 200:259-265) was used
28 to label cells grown on MEF feeder cells. The
29 samples were gently washed three times with ES
30 medium before being incubated with the 1:100
31 secondary antibodies (anti mouse IgG and anti mouse
32 IgM, both Sigma) conjugated to fluorescein

1 isothiocyanate (FITC) at 37°C for 20 minutes. The
2 samples were again washed three times with ES
3 medium and subjected to fluorescence microscopy.
4 For the Oct4 immunostaining hES cells were fixed in
5 3.7% formaldehyde BDH, Coventry, UK for 20 minutes
6 at room temperature followed by incubation in 3%
7 hydrogen peroxide for 10 minutes. The hES cells
8 were permeabilised with 0.2 % Triton x100 (Sigma)
9 diluted in 4% sheep serum (Sigma) for 30 minutes at
10 37°C. The ES colonies were incubated with the
11 primary antibodies (Oct4 from Santa Cruz
12 Biotechnologies, Heidelberg, Germany, final
13 concentration 10 µg/ml for 30 minutes at room
14 temperature. The ES colonies were washed twice
15 with PBS for 5 minutes and then incubated with the
16 secondary antibody (rat anti mouse immunoglobulin
17 (DAKO, Cambridgeshire, UK) used at 1:100 dilution)
18 for 30 minutes at room temperature. After that,
19 hES cells were washed again with PBS, incubated
20 with ABC/HRP solution for 25 minutes at room
21 temperature and washed again with PBS. The
22 detection was carried out by incubation with DAB
23 peroxidase (Enzo Life Sciences, NY) solution at
24 room temperature for 1 minute. Final washes were
25 done with distilled water. The bright field and
26 fluorescent images were obtained using a Zeiss
27 microscope and the AxioVision software (Carl Zeiss,
28 Jena, Germany).

29
30 **Comparison of hES cells-derived fibroblasts with**
31 **human foreskin fibroblasts.** To identify the nature
32 of feeder cells, hESdF were compared with human

1 foreskin fibroblasts (HFF; ATCC, Teddington, UK)
 2 using flow-cytometry analysis. Briefly, hESdF were
 3 harvested using 0.05% Trypsin/0.53M EDTA
 4 (Invitrogen, Paisley, Scotland) and suspended in
 5 staining buffer (PBS +5% FCS) at concentration 10^6
 6 cells/ml. Hundred μ l of the cell suspension was
 7 stained with 0.2 μ g of CD31 (PECAM-1), CD71
 8 (Transferrin receptor), CD90 (Thy-1), and CD106
 9 (VCAM-1) antibodies (all available from BD
 10 Biosciences, Oxford, UK) at 4°C for 20 minutes.
 11 Three washes in staining buffer were carried out
 12 before staining with secondary antibody, goat anti-
 13 mouse Ig-FITC (Sigma, Dorset, UK) used at 1:512
 14 dilution at 4°C for 20 minutes. Cells were washed
 15 again three times and resuspended in staining
 16 buffer before being analysed with FACS Calibur (BD)
 17 using the CellQuest software. 10,000 events were
 18 acquired for each sample and propidium iodide
 19 staining (1 μ g/ml) was used to distinguish live
 20 from dead cells.

21
 22 **Karyotype analysis of hES cells and hES cells-**

23 **derived fibroblasts.** The karyotype of hES cells
 24 and hES cells-derived fibroblasts was determined by
 25 standard G-banding procedure. A suitable protocol
 26 is available at:

27 [http://www.slh.wisc.edu/cytogenetics/Protocols/Stai](http://www.slh.wisc.edu/cytogenetics/Protocols/Staining/G-Banding.html)
 28 [ning/G-Banding.html](http://www.slh.wisc.edu/cytogenetics/Protocols/Staining/G-Banding.html)

29
 30 **Reverse Transcription (RT)-PCR analysis.** The

31 reverse transcription was carried out using the
 32 cells to cDNA II kit (Ambion, Huntingdon, UK)

1 according to manufacturer's instructions. In
2 brief, hES cells were submerged in 100 µl of ice-
3 cold cell lysis buffer and lysed by incubation at
4 75°C for 10 minutes. Genomic DNA was degraded by
5 incubation with DNase I for 15 minutes at 37°C. RNA
6 was reverse transcribed using M-MLV reverse
7 transcriptase and random hexamers following
8 manufacturer's instructions. PCR reactions were
9 carried out using the following primers (Seq ID Nos
10 1 to 12):

11

12 OCT4 (F): 5'-GAAGGTATTCAGCCAAAC-3';
13 OCT4 (R): 5'-CTTAATCCAAAAACCCTGG-3';
14 REX1 (F): 5'-GCGTACGCAAATTAAAGTCCAGA-3';
15 REX1 (R): 5'-CAGCATCCTAAACAGCTCGCAGAAT-3';
16 NANOG (F): 5'-GATCGGGCCCCGCCACCATGAGTGTGGATCCAGCTTG-3';
17 NANOG (R): 5'-GATCGAGCTCCATCTTCACACGTCTTCAGGTTG-3';
18 FOXD3F: 5'-GGAGGGAGGGGGCAATGCAC-3';
19 FOXD3R: 5'-CCCCGAGCTCGCCTACT-3';
20 TERT (F): 5'-CGGAAGAGTGTCTGGAGCAAGT-3';
21 TERT (R): 5'-GAACAGTGCCTTCACCCTCGA-3';
22 GAPDH (F): 5'-GTCAGTGGTGGACCTGACCT-3';
23 GAPDH (R): 5'-CACCACCCTGTTGCTGTAGC-3'.

24

25 Note that (F) and (R) refer to the direction of the
26 primers and designate forward and reverse direction
27 respectively.

28

29 PCR products were run on 2% agarose gels and
30 stained with ethidium bromide. Results were
31 assessed on the presence or absence of the
32 appropriate size PCR products. Reverse

1 transcriptase negative controls were included to
2 monitor genomic contamination.

3
4 **DNA Genotyping of hES cells and hES cells-derived**
5 **fibroblasts.** Total genomic DNA was extracted from
6 both hES cells and hES cells-derived fibroblasts.
7 DNA from both samples was amplified with 11
8 microsatellite markers: D3S1358, vWA, D16S539,
9 D2S1338, Amelogenin, D8S1179, D21S11, D18S51,
10 D19S433, TH01, and FGA (Chen Y et al., 2003, Cell
11 Res. 2003 Aug;13(4):251-63. full paper available at
12 [http://www.cell-research.com/20034/2003-116/2003-4-](http://www.cell-research.com/20034/2003-116/2003-4-05-ShengHZ.htm)
13 [05-ShengHZ.htm](http://www.cell-research.com/20034/2003-116/2003-4-05-ShengHZ.htm)) and analysed on an ABI 377 sequence
14 detector using Genotype software (Applied
15 Biosystems, Foster City, CA).

16
17 **Growth of hES cells on hESdF.** HES-NCL1 cells were
18 grown on γ -irradiated hESdF monolayer (75.000
19 cells/cm²) in ES medium containing Knockout-DMEM
20 (Invitrogen), 100 μ M β -mercaptoethanol (Sigma), 1
21 mM L-glutamine (Invitrogen), 100 mM non-essential
22 amino acids, 10% serum replacement (SR,
23 Invitrogen), 1% penicillin-streptomycin
24 (Invitrogen) and 4 ng/ml bFGF (Invitrogen). ES
25 medium was changed daily. HES cells were passaged
26 every 4-5 days by incubation in 1 mg/ml collagenase
27 IV (Invitrogen) for 5-8 minutes at 37°C or
28 mechanically dissociated and then removed to plates
29 with freshly prepared hESdF.

30
31 **Recovery of hESdF-conditioned medium.** Mitotically
32 inactivated hESdF were cultured in T-25 flask with

1 addition of ES medium for 10 days. hESdF-
2 conditioned medium was collected every day and then
3 frozen at -80°C.

4
5 **Growth of hES cells in feeder-free system using**
6 **hESdF-conditioned medium.** hES cells were passaged
7 and then removed to plates precoated with Matrigel
8 (BD, Bedford, MA) as previously described. ¹⁶ ES
9 media conditioned by hESdF was changed every 48-
10 hours.

11
12 **Cryopreservation of hES cells and hESdF.** To see
13 whether frozen-thawed hESdF still support
14 undifferentiated growth of cryopreserved hES cells,
15 hESdF were frozen at -80°C using FCS supplemented
16 with 10% (v/v) dimethyl sulfoxide (Sigma). Clumps
17 of hES cells were frozen or vitrified using
18 protocol as previously described (see Reubinoff et
19 al., 2001, Hum Reprod 10:2187-2194). Mitotic
20 inactivation by using mitomycin C could
21 alternatively be used.

22
23 **Tumor formation in severe combined immunodeficient**
24 **(SCID) mice (Stefan).** Ten to fifteen clumps with
25 approximately 3000 hES cells in total were injected
26 in kidney capsule, subcutaneously in flank or in
27 the testis. After 21-90 days, mice were
28 sacrificed, tissues were dissected, fixed in Bouins
29 overnight, processed and sectioned according to
30 standard procedures and counterstained with either
31 haematoxylin and eosin or Weigerts stain. Sections

1 were examined using bright field light microscopy
2 and photographed as appropriate.

3

4 All procedures involving mice were carried out in
5 accordance with institution guidelines and
6 institution permission.

7

8 **Statistical analysis.** Cell numbers of Day 6 and Day
9 8 ICMs were compared using Wilcoxon rank-sum test.
10 The data are presented as mean \pm standard
11 deviation.

12

13 ***In vitro* differentiation of hES cells.** Colonies of
14 hES-NCL1 passage 21 were grown in feeder-free
15 conditions in ES medium. After 5 to 14 days
16 spontaneous differentiation was observed and
17 differentiated cells were passaged and cultured
18 under same conditions. Cells were fixed in 4%
19 paraformaldehyde in PBS (Sigma) for 30 minutes and
20 then permeabilised for additional 10 minutes with
21 0.1% Triton X (Sigma). The blocking step was 30
22 minutes with 2% FCS in PBS. Cells were incubated
23 with antibody against nestin (1:200; Chemicon) or
24 human alpha smooth muscle actin (1:50; Abcam,
25 Cambridge, UK) for additional 2 hours. Each
26 antibody was detected using corresponding secondary
27 antibodies conjugated to FITC. The nuclei of cells
28 were stained using propidium iodide for 5 minutes.

29

30 **Results**

31 Traditionally early blastocysts (Day 6) have been
32 used for the derivation of human ES cell line. We

1 developed a three - step culture system (see
2 Materials and Methods) which supports successfully
3 the development of late (Day 8) blastocysts.
4 Analysis of cell numbers of ICMs revealed that Day
5 8 blastocysts possess significantly ($P < 0.01$) more
6 ICM cells than Day 6 blastocysts (51.3 ± 9.6 vs.
7 36.8 ± 11.9 , respectively). In view of this result
8 we used day 8 blastocysts to derive human ES cell
9 lines. Of the 11 Day 2 donated embryos, 7 (63.6%)
10 blastocysts developed to Day 6. All 7 of these
11 blastocysts expanded or hatched on Day 8 after
12 transfer to G-BRLC medium. After isolation of ICMs
13 by immunosurgery, 3 primary hES cell colonies
14 showed visible outgrowth and one stable hES cell
15 line (ICL-NCL1) was successfully derived (Figs. 1C-
16 E).

17
18 When the hES cells were cultured in the absence of
19 feeder cells they spontaneously differentiated into
20 fibroblast-like cells, ie. long, flat cells with
21 elongated, condensed nucleus. We confirmed that
22 the differentiated cells were fibroblasts by
23 staining with a specific antibody to fibroblast
24 surface protein (AFSP) (Fig. 2C and D). Karyotyping
25 of the hES cells and hES cells-derived fibroblasts
26 revealed that both samples are normal female ($46 +$
27 XX , Figs. 2E and F). Microsatellite analysis
28 revealed that the hES cells and hES cells-derived
29 fibroblasts are indistinguishable from each other
30 and should be considered as autogenic (see Fig. 2G,
31 2H). We now have several batches of fresh and
32 frozen/thawed serially expanded hES cells-derived

1 fibroblasts which support hES cell culture even
2 after the twelfth passage but they are optimal
3 between second and eighth passages. Flow-cytometry
4 (Fig. 7) revealed that very few cells showed
5 expression of mesenchymal cell specific markers
6 CD106 (V-CAM1) and CD71 (transferring receptor) and
7 none expressed the endothelial specific cell marker
8 CD31 (PECAM-1). On the contrary, 94% and 82% of the
9 hESdF cells were stained with the CD44 and CD90
10 (THY-1) antibodies, respectively. Both antibodies
11 were also presented in human foreskin fibroblasts
12 (HFF; Fig. 7).

13
14 The hES-NCL1 line has been cultured on hES cell
15 derived fibroblasts (hESdF) for over 35 passages
16 and on Matrigel with hESdF conditioned medium for
17 13 passages. We found that hES cell colonies grown
18 on hES cell derived fibroblasts were dense, compact
19 and suitable for mechanical passaging with typical
20 morphology of hES cells (Fig. 4). Characterisation
21 studies demonstrated that hES cells cultured on hES
22 cells-derived fibroblasts or Matrigel with addition
23 of hESdF-conditioned medium expressed specific
24 surface markers: GTCM2, TRA1-60 and SSEA4, and
25 (Fig. 4A-H) and were positive for the expression of
26 *OCT-4*, *NANOG*, *FOXD3*, *REX-1* and *TERT* by RT-PCR (Fig.
27 5A). Expression of TG343 was also found in hES
28 cells grown on mouse feeder cells, and whilst not
29 tested in the hESdF grown cells would be expected
30 to be present. The fibroblast-like cells also
31 expressed the telomerase reverse transcriptase
32 (*TERT*) and *REX1* in early passages but none of the

1 other ES cell specific markers. Human ES cells
2 grafted into SCID mice consistently developed into
3 teratomas demonstrating the pluripotency of hES-
4 NCL1 cells grown on hESdF. Teratomas were primarily
5 restricted to the site of injection and their
6 histological examination revealed advanced
7 differentiation of structures representative of all
8 three embryonic germ layers, including cartilage,
9 skin, muscle, primitive neuroectoderm, neural
10 ganglia, secretory epithelia and connective tissues
11 (Fig. 6). When hES-NCL1 cells were cultured in
12 absence of feeders and Matrigel, spontaneous
13 differentiation into neuronal (Fig. 8A) and smooth
14 muscle (Fig. 8B) cells was observed.
15

1 / 8

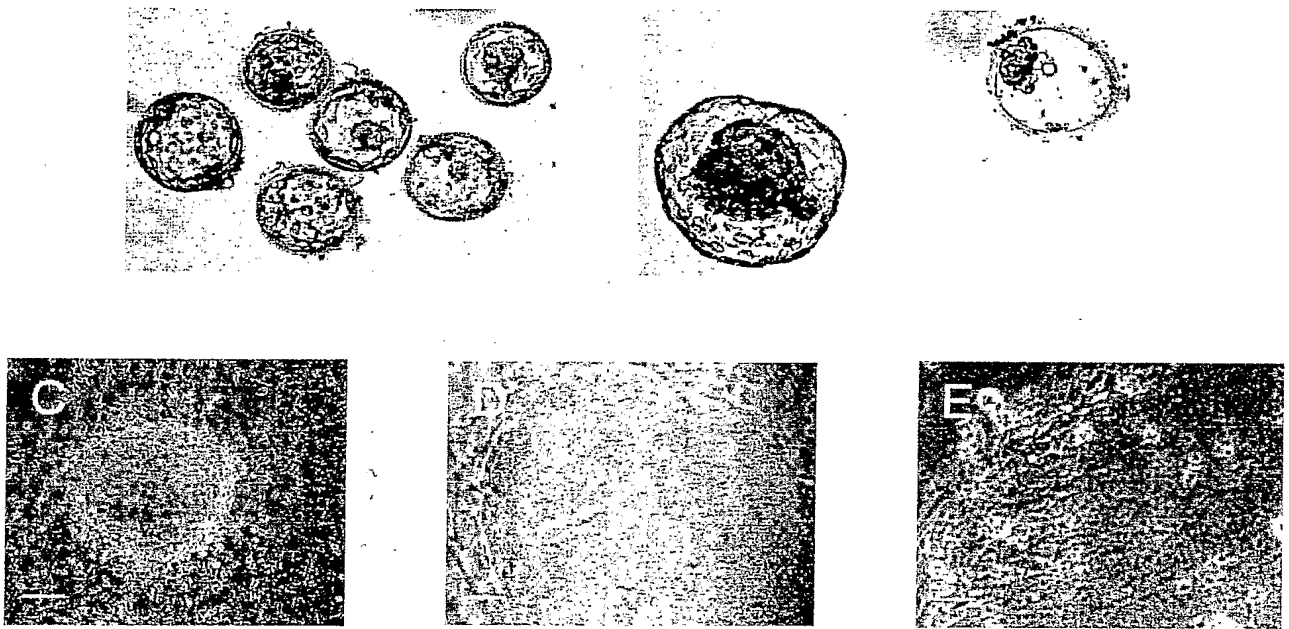


Fig. 1



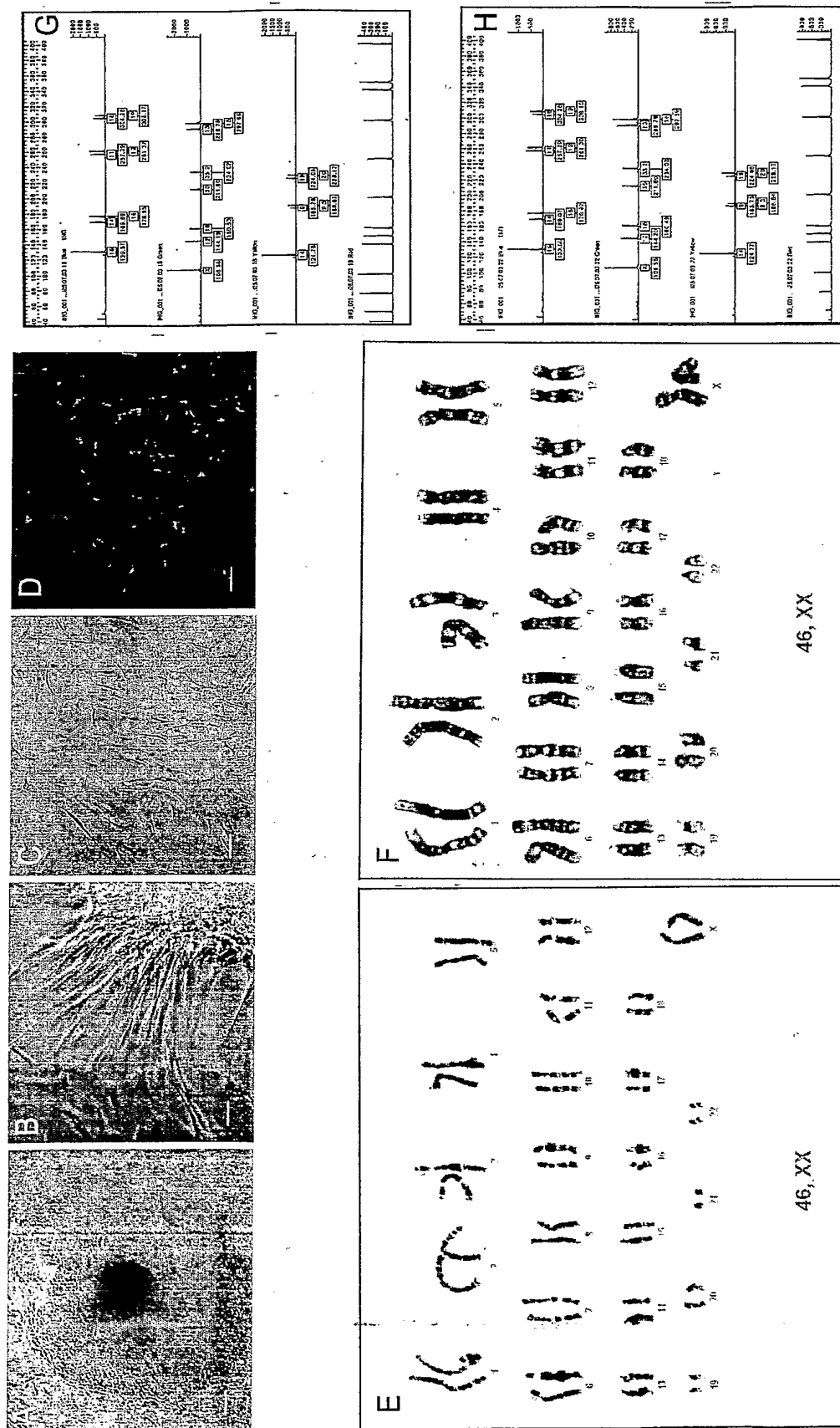


Fig. 2



3 / 8

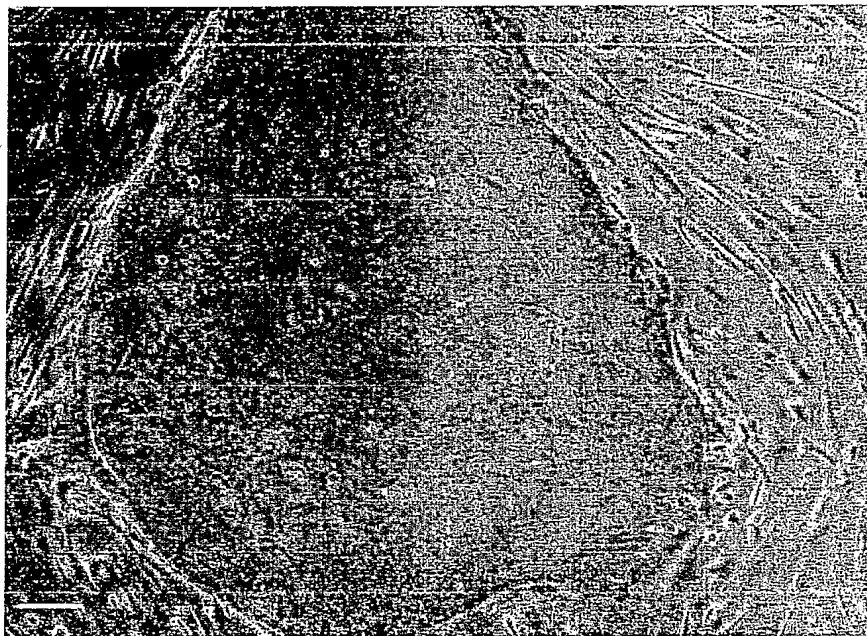


Fig. 3



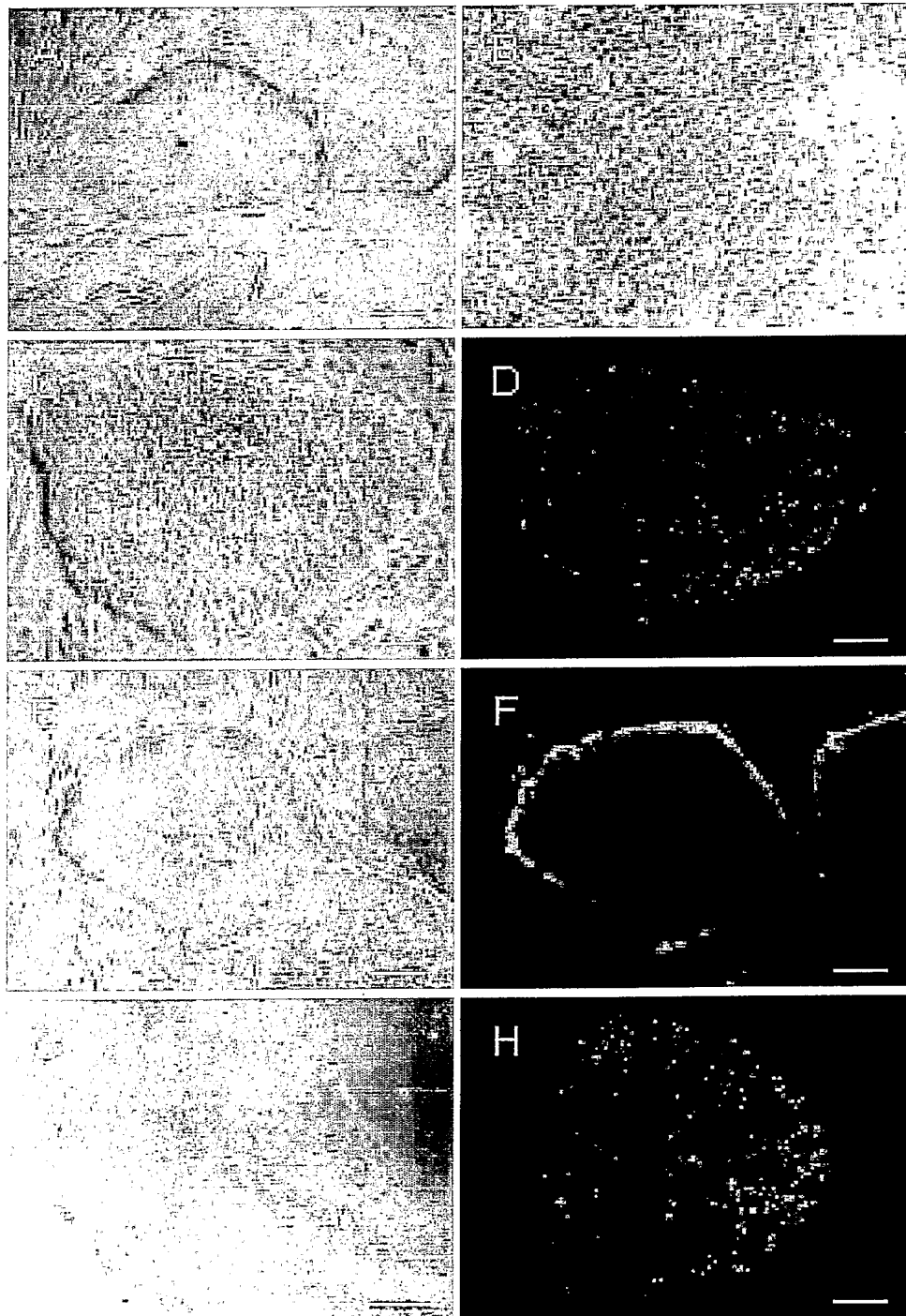
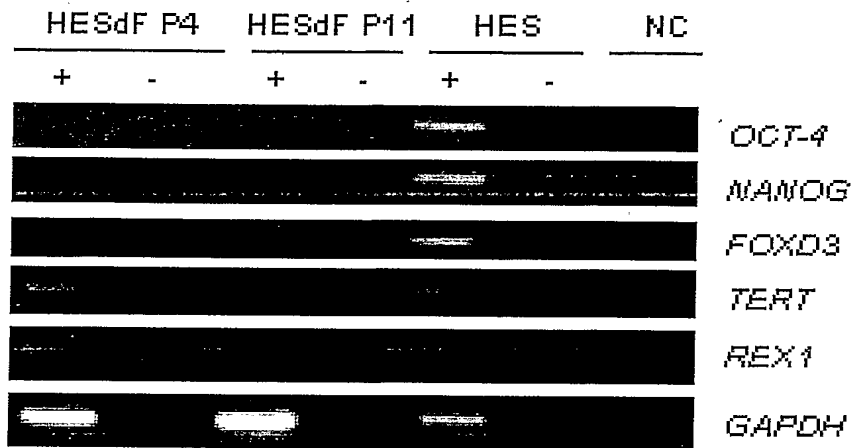


Fig. 4



A



B

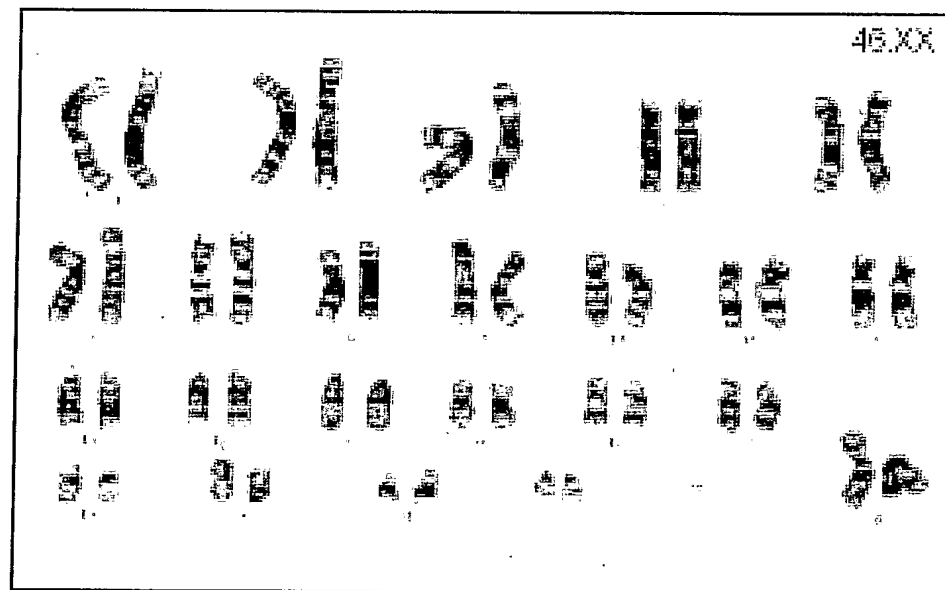


Fig. 5



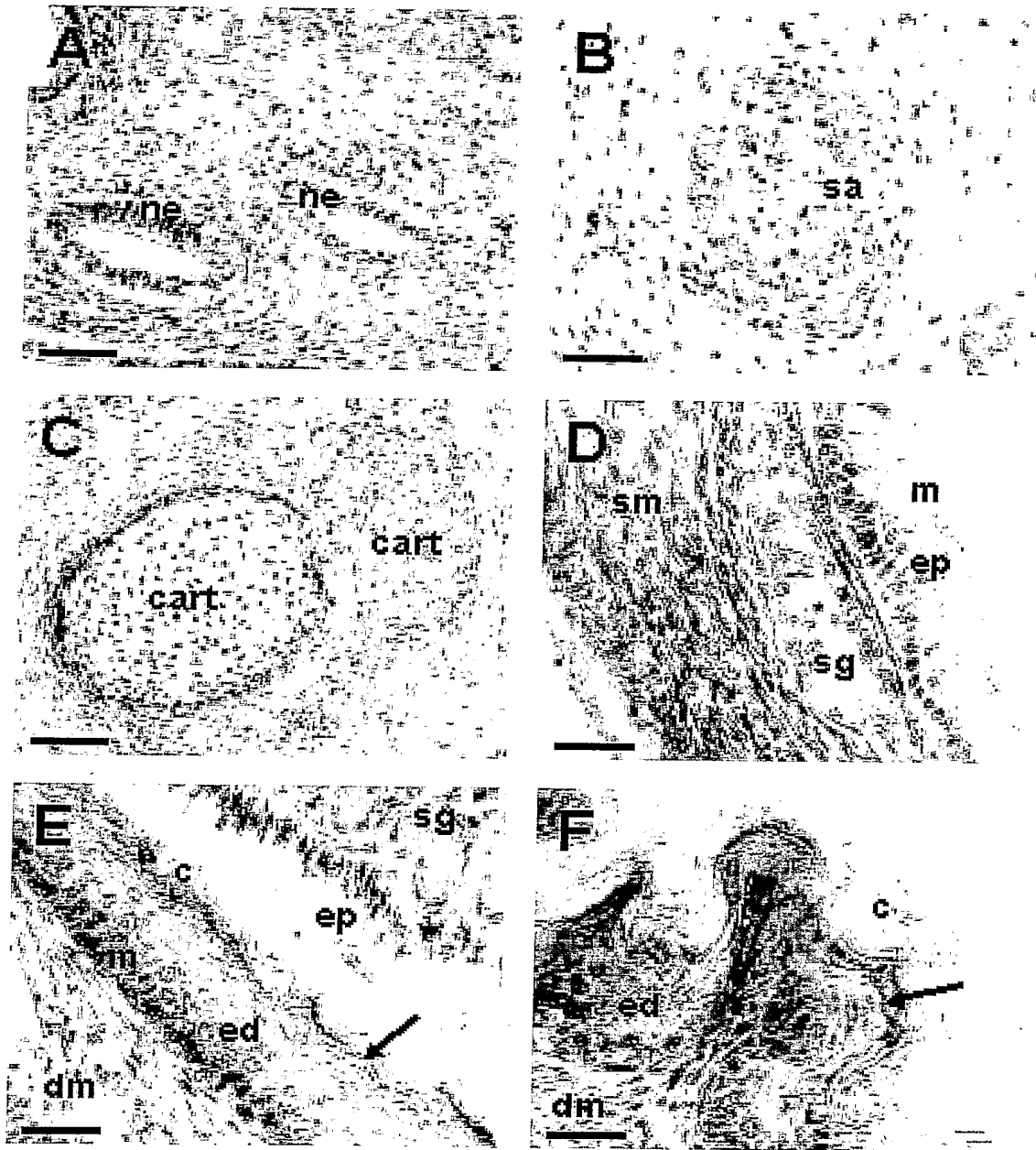


Fig. 6



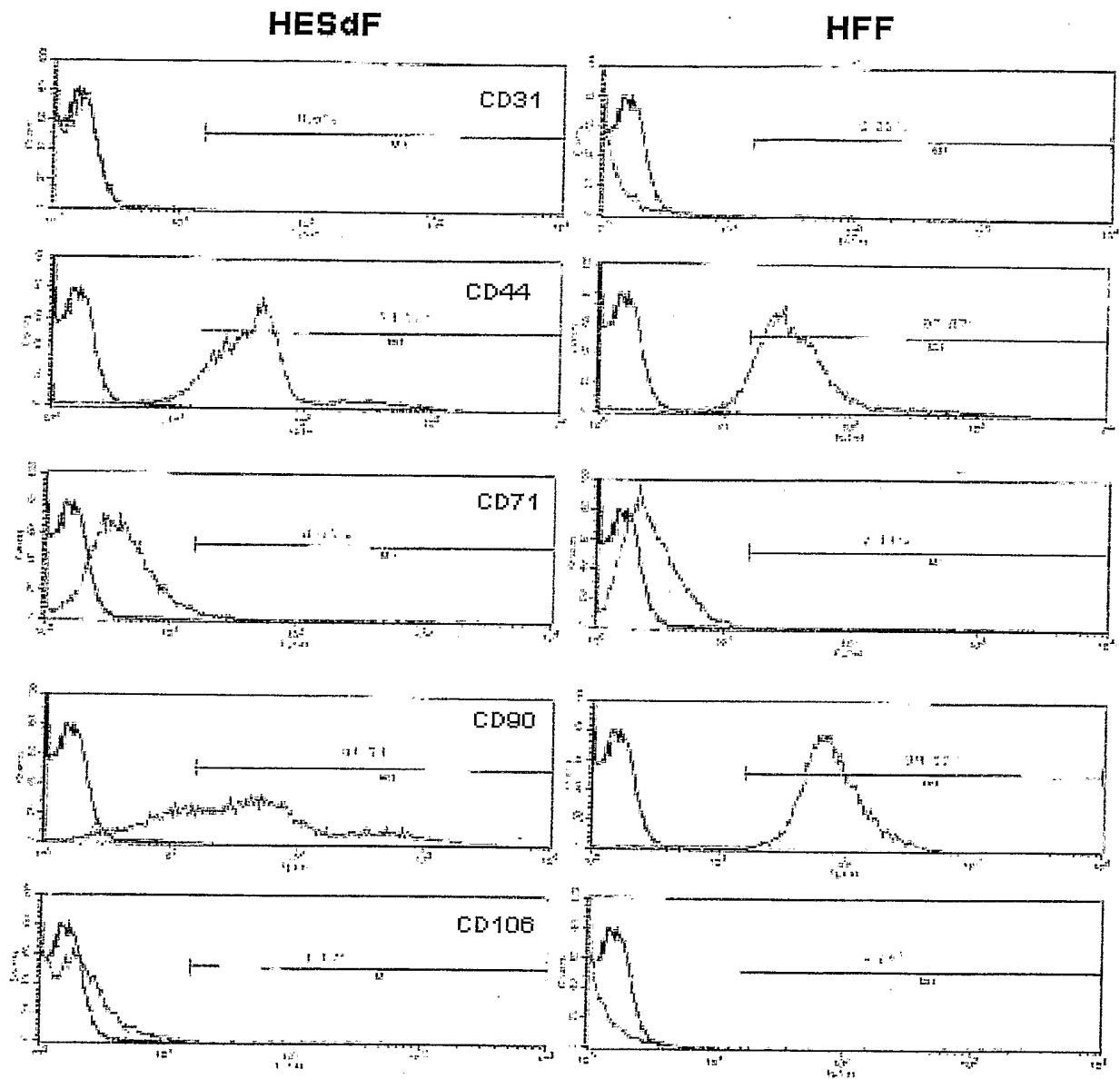


Fig. 7



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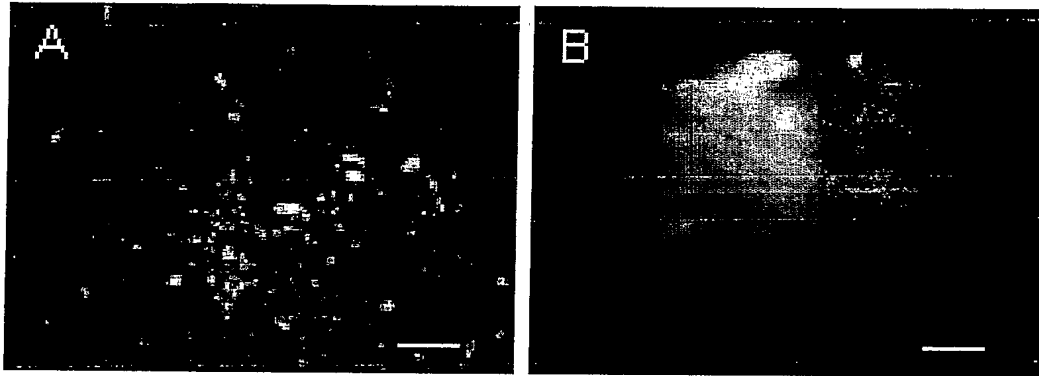


Fig. 8

